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Herbimycin A Suppresses the Reduction of Gap-junctional Intercellular Communication Induced by Tumor-promoting Phorbol Ester in 3T3-L1 Cells

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We have examined the suppressive effect of herbimycin A on the reduction of gap-junctional intercellular communication that is induced by a tumor-promoting phorbol ester in 3T3-L1 cells. Most cells in growth arrest participated in dye-coupling, as evaluated by the transfer between cells of a fluorescent dye (Lucifer Yellow CH). Treatment of cells with 0.25 μg/ml herbimycin A slightly enhanced the dye-coupling. This enhancement required treatment for periods as long as 24 h. Addition of 100 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA) caused a rapid reduction of dye-coupling. However, addition of TPA did not suppress dye-coupling in cells pretreated for more than 24 h with herbimycin A. Pretreatment of cells for less than 6 h with herbimycin A did not suppress the TPA-induced reduction of dye-coupling. These results suggest that herbimycin A suppresses the reduction of gap-junctional intercellular communication that is induced by TPA through enhancement of the ability of the cells to participate in gap-junctional intercellular communication.

Key words: phorbol ester — Intercellular communication — Dye coupling — Herbimycin — 3T3-L1 cells

Intercellular communication via gap junctions has been postulated to play a crucial role in regulation of cell growth and differentiation.[1-3] Moreover, reduction of gap-junctional intercellular communication by the potent tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) seems to be related to the promotion of tumors, leading, in some systems, to the induction of skin tumors.[4-6] Cyclic AMP inhibits TPA-induced reduction of gap-junctional intercellular communication.[7] Inhibition of TPA-induced reduction in communication might be important for the prevention of tumor promotion.

TPA also modulates cell-to-substratum interactions and organization of actin filaments.[8-10] Herbimycin A, isolated from the filtrate of medium used for the culture of *Streptomyces* sp. MH 237-CF-8,[11] suppresses the biological effects of tumor-promoting phorbol esters on intercellular contacts, cell-to-substratum contacts, the organization of actin filaments and cell shape.[8] Herbimycin A causes a reversion from transformed morphology to normal morphology in rat kidney cells infected with Rous sarcoma virus.[11] These actions of herbimycin A seem to result from the modulation of cell-to-substratum interactions, since herbimycin A stimulates the expression of fibronectin.[12] The interaction of cells with an adequate substratum is required for the maintenance of intercellular communication[13] and, therefore, we postulated that herbimycin A might suppress the TPA-induced reduction in intercellular communication.

Gap-junctional intercellular communication develops effectively during growth arrest of fibroblastic cell lines, while it does not develop in epithelial cell lines. In the present study, we investigated the effects of herbimycin A on the TPA-induced reduction of intercellular communication in growth-arrested 3T3-L1 cells. Herbimycin A stimulates gap-junctional intercellular communication and suppresses the TPA-induced reduction in such communication.

MATERIALS AND METHODS

Cells and chemicals Swiss mouse embryo 3T3 fibroblasts, clone L1 (3T3-L1), (about 1×10^4 cells per culture dish of 35 mm in diameter) were cultured for 2 days in Dulbecco's modified Eagle's (DME) medium supplemented with 10% calf serum (Gibco Laboratories; Grand Island, NY). Growth arrest of 3T3-L1 cells was achieved in monolayers by culture of cells in DME medium with 2% calf serum for 4 days.

Herbimycin A was isolated from the filtrate of medium used for the culture of *Streptomyces* sp. MH237-CF-8.[11] TPA was purchased from Sigma (St. Louis, MO). The drugs were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was less than 0.1% in solutions of 100 ng/ml TPA.

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Measurement of dye-coupling The fluorescent dye Lucifer Yellow CH was microinjected pneumatically into individual cells in a monolayer of 3T3-L1 cells. At least 30 microinjections were carried out per dish of cultured cells. Dye was transferred from an injected cell to neighboring coupled cells within 5 min. The cultures were fixed with 10% formalin 5 min or more after the final injection. The number of dye-recipient cells per injection of dye was determined under an inverted phase-contrast microscope equipped with fluorescence apparatus. The extent of dye-coupling was expressed in terms of two indices: incidence of dye-coupling (number of dye-couplings/total number of injections) and the mean number of dye-recipient cells in cases where coupling of cells occurred after injection.

RESULTS

3T3-L1 cells in growth arrest showed evidence of well-developed gap-junctional intercellular communication (Fig. 1A). The mean number of dye-recipient cells was 7.7 ± 3.6 (mean $\pm$ standard deviation, $n = 135$) for dye-coupled cells (91.8% of total injections). Treatment of the cells for 24 h with 0.25 μg/ml herbimycin A resulted in enhancement of dye-coupling (Fig. 1B) and slight morphological changes (Fig. 2B). Dye-coupling decreased after a 1-h treatment with 100 ng/ml TPA (Fig. 1C). The reduction in dye-coupling by TPA was observed both in morphologically changed cells and in unchanged cells (Fig. 2C). Addition of TPA failed to

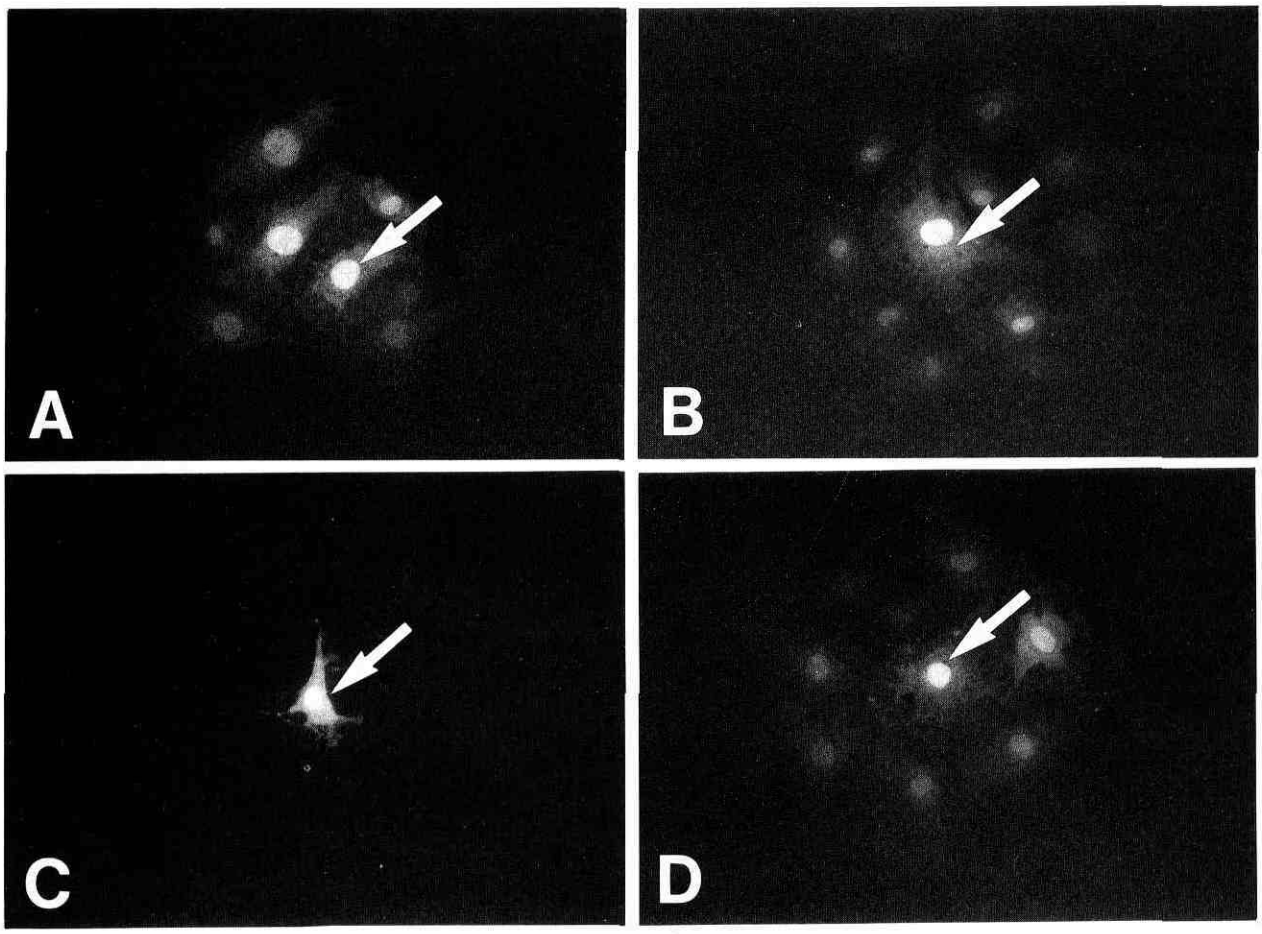

Fig. 1. Effects of TPA on dye-transfer in 3T3-L1 cells pretreated with herbimycin A. Dye was transferred from individual cells injected with Lucifer Yellow to coupled neighboring cells in control (A). Treatment for 24 h with 0.25 μg/ml herbimycin A caused a marked increase in the transfer of dye (B). Addition of 100 ng/ml TPA for 1 h to cells in growth arrest caused a rapid decrease in dye-transfer (C), but the addition of TPA did not cause suppression of dye-transfer in the cells pretreated for 24 h with 0.25 μg/ml herbimycin A (D). Arrows indicate the cells injected with the dye. $\times 175$.

reduce dye-coupling (Fig. 1D) and to induce morphological change (Fig. 2D) in cells pretreated for 24 h with herbimycin A.

The enhancement of dye coupling with herbimycin A was dependent on the duration of treatment with herbimycin A (Figs. 3 and 4). Treatment of the cells for 30 h significantly increased the incidence of dye-coupling ($P<0.05$) and treatment for 24 h significantly increased the number of dye-recipient cells ($P<0.01$), but treatment for less than 6 h did not have this effect.

The incidence of dye-coupling was not decreased after a 1-h incubation with TPA in the case of cells pretreated for 24 h with herbimycin A (Fig. 3). The suppressive effect of herbimycin A on the TPA-induced reduction in communication was dependent on the duration of pretreatment with herbimycin A. Pretreatment for less than 6 h with herbimycin A did not suppress TPA-induced reduction of dye-coupling. There were significant differences between the parameters of coupling for cells treated with herbimycin A alone and those treated with the combination of herbimycin A and TPA ($P<0.001$).

The number of dye-recipient cells was reduced after a 1-h incubation with TPA in the case of cells pretreated for 24 h with herbimycin A (Fig. 4). There was a significant difference between the numbers obtained with cells before and after treatment with TPA ($P<0.005$). However, the reduced value was similar to that for control cells and was still significantly higher than correspoding

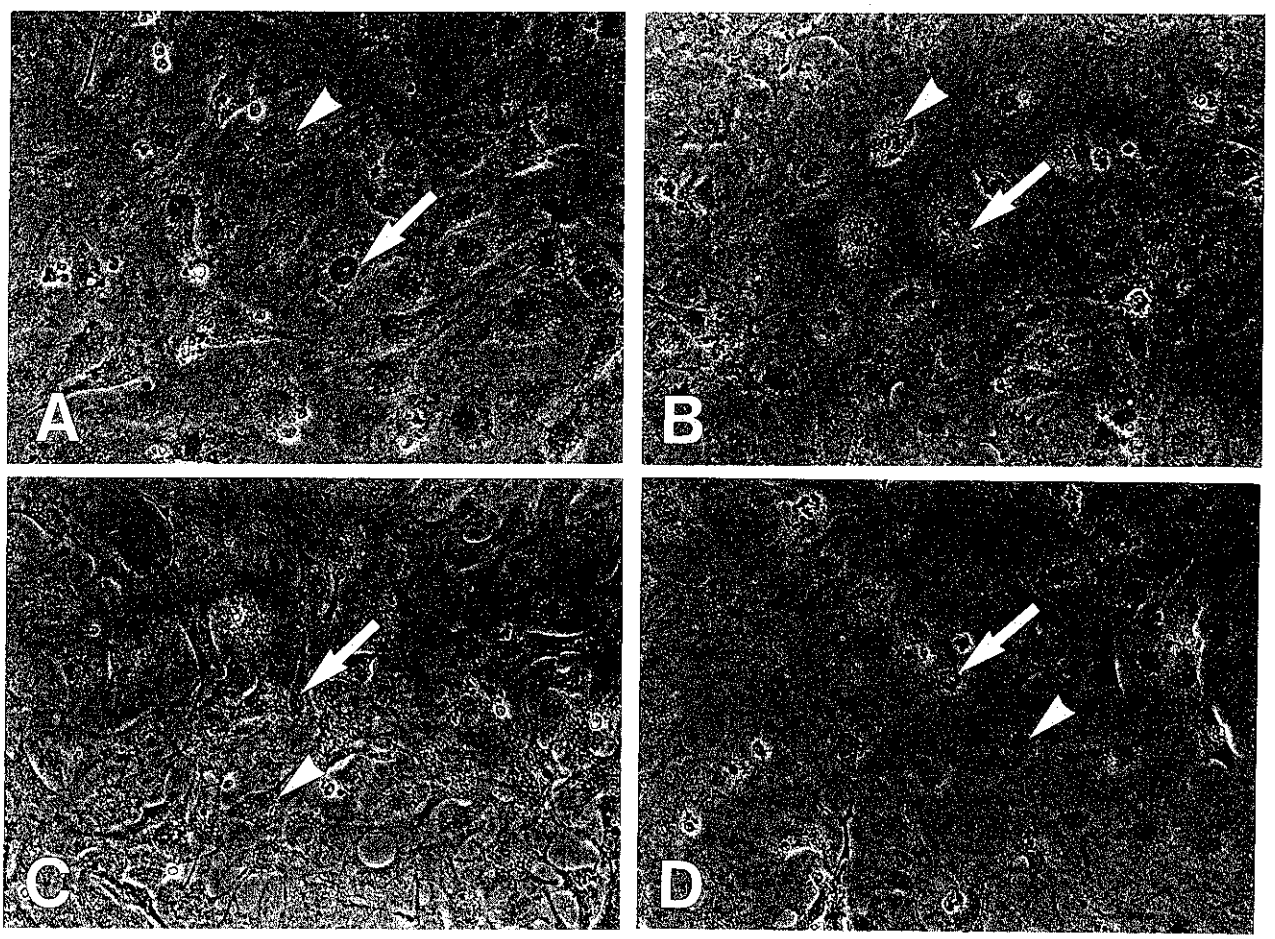

Fig. 2. Effects of herbimycin A and TPA on cell morphology in growth-arrested 3T3-L1 cells. The same field of corresponding cultures in Fig. 1 is shown by phase-contrast micrography. Cells in growth arrest were polygonal in shape when in contact with each other (A). Treatment for 24 h with 0.25 μg/ml herbimycin A caused a slight increase in spreading (B). Addition of 100 ng/ml TPA for 1 h to cells in growth arrest caused a slight change in cell shape (C), but the addition of TPA did not cause any morphological changes in cells pretreated for 24 h with 0.25 μg/ml herbimycin A (D). Arrows indicate the cells injected with the dye. Arrowheads indicate the cells with typical morphology. $\times$175.

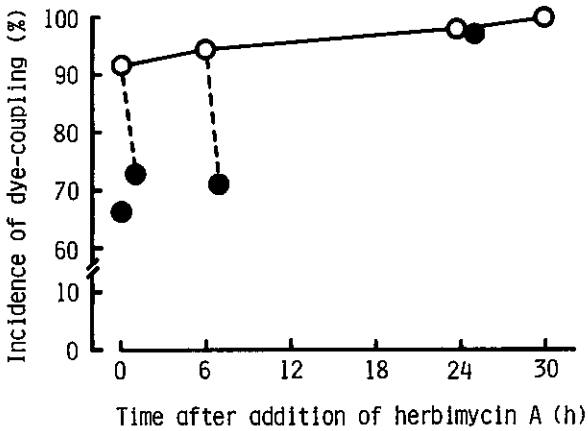

Fig. 3. Effects of herbimycin A and TPA on the incidence of dye-coupling in a population of growth-arrested 3T3-L1 cells. Cells in growth arrest were incubated with 0.25 μg/ml herbimycin A for various periods, as indicated. 12-O-Tetradecanoylphorbol-13-acetate (TPA) was added for 1 h at the various times indicated after the addition of herbimycin A. Then the incidence of dye-coupling (%) was calculated from the results (n=38–147) obtained before (○) and after the addition of TPA (●).

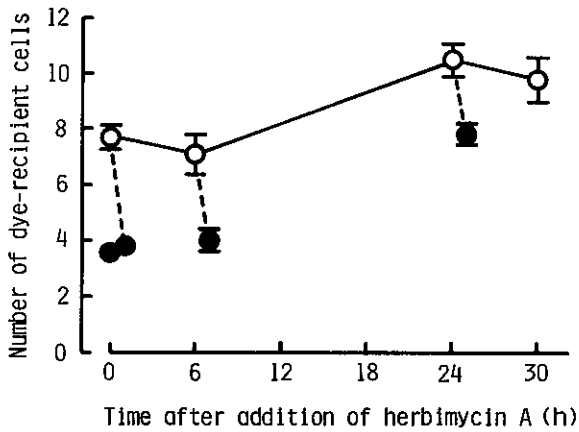

Fig. 4. Effects of herbimycin A and TPA on the number of dye-recipient cells in a growth-arrested population of 3T3-L1 cells. Cells in growth arrest were incubated with 0.25 μg/ml herbimycin A for the periods of time indicated. TPA was added for 1 h at the various times indicated after the addition of herbimycin A. The number of dye-recipient cells per individual coupled cell was calculated from the results obtained before (○) and after addition of TPA (●), and is given as the mean ±standard error (n=34–135). Bars representing the standard error are shown except in cases where the standard error was less than the size of the symbol.

Table I. Effect of TPA on Dye-coupling of 3T3-L1 Cells Pretreated with Herbimycin A

	% Incidence of dye-coupling	Number of dye-recipient cells
Control	91.8 (135/147)	7.7±0.3 (135)
Herbimycin A	100 (48/48) — *	9.8±0.8 (48) — **
TPA	51.5 (53/103) — **	2.8±0.2 (53) — ***
Herbimycin A + TPA	94.1 (64/68) — ***	4.4±0.3 (64) — ***

Cells in growth arrest were pretreated for 24 h with 0.25 μg/ml herbimycin A and then treated for a further 6 h with 100 ng/ml TPA. Dye-coupling was determined 30 h after the addition of herbimycin in the absence or presence of TPA, and the results are expressed as the incidence of dye-coupling (number of dye-couplings/total number of injections) or as number of dye-recipient cells per individual coupled cell (mean±standard error). The numbers in parenthesis on the left indicate the number of dye-couplings per total number of injections. The numbers in parenthesis on the right are numbers of coupled cells. *, ** and *** indicate significant differences ($P<0.05$, $P<0.01$, $P<0.001$, respectively).

values for the cells treated for less than 6 h with herbimycin A ($P<0.001$).

Treatment of cells for 6 h with 100 ng/ml TPA markedly reduced dye-coupling (Table I). Pretreatment with 0.25 μg/ml herbimycin A suppressed the TPA-induced decrease in the incidence of dye-coupling and partially, but significantly, suppressed the TPA-induced decrease in the number of dye-recipient cells.

DISCUSSION

Herbimycin A enhanced the dye-coupling and spreading of growth-arrested 3T3-L1 cells. This enhancement required a relatively long period of treatment. Such a time-dependence has always been observed in the case of other effects of herbimycin A, namely, the restoration of non-transformed morphology,[11] enhancement of the ex-

pression of fibronectin,[12] and the suppression of TPA-induced changes in the shape of colonies of cells.[8] It appears that the effect of herbimycin A may be the result of changes in cell functions which occur over a rather long period of time.

Most of the effects of herbimycin A can be reasonably explained as being the results of stimulated expression of fibronectin.[12] Restoration of non-transformed morphology and suppression of TPA-induced changes in colony shape seem to be closely related to modulations of the interaction between cells and substratum via fibronectin. The synthesis of fibronectin is stimulated by cyclic AMP.[14] Cyclic AMP has, thus far, only been found to enhance gap-junctional intercellular communication between cultured cells and its stimulative effect also requires a rather long period of treatment.[15–17] Moreover, cyclic AMP also suppresses the TPA-induced reduction in gap-junctional intercellular communication[7] and changes in colony shape.[18] Taken together, it seems possible that the enhancement of dye-coupling and suppression of TPA-induced uncoupling by herbimycin A might be closely related to the stimulated synthesis of fibronectin through the elevation of intracellular concentration of cyclic AMP.

The *src* gene product or *src* protein kinase is one of the downregulators of gap-junctional intercellular communication.[19–21] Herbimycin A suppresses *src* protein kinase.[11] A suppressive effect of herbimycin A on *src* protein kinase might be involved in the suppression of TPA-induced uncoupling, because TPA-induced uncoupling is caused by the activation of protein kinase C.[22] Such action of herbimycin A should not be ignored, although suppression of dye-coupling by *src* protein kinase is rapidly blocked by TMB-8.[21]

The inhibitory action of herbimycin A can be observed upon many of the changes induced by TPA: uncoupling, dissociation of cells, morphological changes, changes in interactions between cells and the substratum, and changes in the organization of actin filaments.[8] Such changes induced by TPA can be summarized as the disorganization of tissue.[9] Herbimycin A apparently suppresses the TPA-induced disorganization of tissue. Since the TPA-induced promotion of tumors can be suppressed by herbimycin A,[23] changes in gap-junctional intercellular communication, in addition to the various mechanisms that induce the disorganization of tissue, may play some role in the promotion of tumors by TPA.

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